

# The rise and fall of methanotrophy following a deepwater oil-well blowout

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## 6 **Methods**

### 7 *Study Sites:*

8 A total of 1128 water column samples were collected during ten research cruises to the  
9 Gulf of Mexico spanning March (the background sampling cruise) through December  
10 2010, on board of the R/V Pelican: 1<sup>st</sup> Mar. 2010 – 7 Mar. 2010, 5<sup>th</sup> May 2010 – 15 May  
11 2010 and 20<sup>th</sup> June 2010 – 26 June 2010; R/V F.G. Walton Smith: 25<sup>th</sup> May 2010 – 6  
12 June 2010; R/V Nancy Foster: 30 June 2010 – 18 July 2010; R/V Oceanus and R/V Cape  
13 Hatteras: 21<sup>st</sup> Aug. 2010 – 16 Sept. 2010; R/V Arctic Sunrise: 20<sup>th</sup> Sept. 2010 – 28 Sept  
14 2010; and R/V Atlantis: 8<sup>th</sup> Nov. 2010 – 3<sup>rd</sup> Dec. 2010 (See On-Line Supplementary  
15 Table). The pre-spill samples were collected from above a fracture zone at a natural  
16 hydrocarbon seep, Mississippi Canyon block 118, in March 2010, roughly a month  
17 before the Macondo Blowout began. It is important to note that the pre-spill data does not  
18 reflect the microbial abundance of an inactive site that lacks natural seepage inputs rather  
19 the data reflects a site impacted by low rates of natural seepage.

20

### 21 *Sample collection:*

22 A CTD-Niskin rosette system was used to obtain hydrographic profiles throughout the  
23 water column. Niskin bottles were triggered at specific depths and upon return to the  
24 surface, each bottle was individual samples were collected for dissolved gas

concentration determination, rates of aerobic methane oxidation, nutrient and metal concentration determination, and microbial molecular analysis (see below).

*Methane concentration:*

Samples for dissolved alkane quantification were collected as soon as the CTD rosette was secured on deck, as described previously by Joye et al.<sup>31</sup> with some modifications between cruises. Concentration of C<sub>1</sub> to C<sub>5</sub> alkanes were determined using headspace extraction (May through or a modified sonication/vacuum extraction technique (August through December)<sup>32</sup>, followed by gas chromatography. A sub-sample (0.25 to 1 mL) gas sample was injected into a gas chromatograph (model 8610C, SRI, California) equipped with a flame ionization detector<sup>31</sup>. A temperature ramp was employed to elute C<sub>3+</sub> alkanes. Concentrations were calculated by comparison to a certified mixed alkane standard (C<sub>1</sub> to C<sub>5</sub>, including both *n*- and *i*- C<sub>4</sub>) (Scott Specialty Gases<sup>®</sup>).

*Aerobic methane oxidation rates:*

Water column aerobic methane oxidation rates were measured using a tritiated (<sup>3</sup>H) CH<sub>4</sub> radiotracer technique<sup>33,34</sup>. Reactions were done in triplicates in gas-tight glass vials. A 100 µl aliquot of the C<sup>3</sup>H<sub>4</sub> tracer solution was injected into each replicate yielding a tracer activity of 2 kBq. Killed controls were achieved by treating a sample with 3.7 % formaldehyde, to arrest microbial activity, prior to tracer addition. The samples were incubated at *in situ* temperature for 48 to 72 hours; linearity of activity was confirmed by time series. Reactions were terminated by adding 20% (vol:vol) of pure ethanol to each

vial. Labeled  $C^3H_4$  was removed by purging the sample with hydrated air for at least 25 minutes. Scintillation cocktail (ScintiSafe Gel<sup>®</sup>) was then added to an aliquot of the sample and  $^3H_2O$  produced was quantified using a Beckman 6500 liquid scintillation counter.

*Methane stable carbon isotopic signature:*

Water samples for methane stable carbon isotopic ( $\delta^{13}C$ ) analyses (May and July) were collected into 125 mL serum vials. A headspace sub-sample from each vial was injected into a Finnegan Mat Delta V Isotope Ratio Mass Spectrometer coupled to a Hewlett Packard gas chromatograph with a poraplot capillary column. Samples were cryo-focused as described in Chanton and Liptay (2000)<sup>35</sup>, and reported relative to Vienna Pee Dee Belemnite (VPDB) standard.

*Nutrients/Biogeochemistry:*

Fixation and analysis of water samples for quantifying dissolved ammonium ( $NH_4^+$ ), inorganic carbon (DIC or  $HCO_3^-$ ), nitrate plus nitrite ( $NO_x^-$ ), and phosphate ( $PO_4^{3-}$ ) followed previously described methods<sup>36</sup>. Samples for dissolved trace elements were collected using trace element cleaned, rosette-mounted, teflon-coated, external spring Niskin and Go-Flo bottles. Samples were cleanly syringe-filtered<sup>37</sup>. Metals were isolated from a small volume (3 mL) of sample using Mg-induced co-precipitation with added enriched  $^{57}Fe$  and  $^{65}Cu$ <sup>38</sup> and then analyzed using isotope-dilution by sector-field inductively coupled plasma-mass spectrometry.

*DNA extraction:*

Water samples for DNA extraction were filtered through a 0.22 µm Sterivex filter (Millipore) and stored frozen until extraction. The filter was extracted using the Ultra Clean Soil DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions with few modifications. Two samples used for *pmoA* clone libraries (see section below) were extracted using different phenol:chloroform DNA extractions protocols; sample C40-6 was extracted following Vetriani *et al.* (1999)<sup>39</sup> while sample C59-9 was extracted according to Adams *et al.* (2013)<sup>40</sup>. Similar results from *pmoA* clone libraries were obtained, regardless of the extraction method used, see Fig. 3.

*qPCR amplification:*

Amplification of Type I relatives OPU1 and OPU3 was achieved with primer set putative\_16S\_OPU1F (5'-CAATGCCGCGTGTGTGAA-3') and putative\_16S\_OPU1R (5'-CCTCTCTTCCCCCGACTGAA-3') for OPU1, and primer set putative\_OPU3F (5'-AGCACTTTCAATTGGGAGGAAA-3') and putative\_OPU3R (5'-GCCGGTGCTTCTTCTAAAGGT-3') for OPU3<sup>41</sup>. The unusual *pmoA* sequences were originally detected using monooxygenase intergenic spacer analysis (hereafter, MISA)<sup>42</sup> and full length *pmoA* sequences were recovered subsequently following the strategy reported in Tavormina 2013<sup>42</sup>. The MISA assay was validated on cultured strains as reported in Tavormina *et al.*<sup>42</sup>. qPCR primers were developed from the full length sequence using Primer3 software <http://frodo.wi.mit.edu/primer3/>.

93 On linearized, CsCl-purified plasmid templates, these primer pairs demonstrate >95%  
 94 amplification efficiency (as measured over 5 orders of magnitude template  
 95 concentrations). Cross reactivity between target plasmid templates is <1%, however these  
 96 primers may amplify related sequences in complex environmental samples. Quantitative  
 97 PCR reactions were performed in a StepOne Real Time PCR System (Applied  
 98 Biosystem), in a reaction consisting of 12.5µl of Power SYBR® Green PCR Master Mix,  
 99 1µl of template DNA (1-6 ng/µl), 0.625µl of BSA (10 ng/µl), 1.25µl of each primer  
 100 (0.5µM final concentration), and water to a final volume of 25µl. qPCR programs  
 101 consisted in a 95°C initial denaturation for 10 min, followed by 40 cycles of 95°C for 15  
 102 sec and a one step annealing/extension of 1 min at 57°C for Type I methanotrophs 16S  
 103 rRNA gene, as well as for OPU1 and OPU3, or 1 min at 60°C in the case of Type II  
 104 methanotrophs. The limit of detection for the qPCR assays was estimated to be 30 copies  
 105 per reaction for Type I, 50 copies per reaction for Type II, 200 copies per reaction for  
 106 OPU1, and 56 copies per reaction for OPU3. All the results were standardized to the  
 107 volume (per mL) of seawater the DNA was extracted from.  
 108  
 109 Enumeration of *pmoA* phylotypes was performed in an ABI 7300 real time PCR system  
 110 with the following conditions. The OPU3 *pmoA* phylotype was performed using primers,  
 111 Taqman probe, and conditions as previously reported<sup>42</sup>. Primers and Taqman probe  
 112 specific to the OPU1 *pmoA* phylotype (primers *pmoA*\_OPU1qPCR\_242f: 5'-  
 113 TTACCCCGATCATGCTGGTT-3' and *pmoA*\_OPU1qPCR\_312r: 5'-  
 114 GATTCTGAAGTGTTCCTCAAACGA-3'. Probe: 5'-TTCCCAGCCGCTGTTTCAGGCA-  
 115 3') and primers specific to the novel *pmoA* phylotype (primers *pmoA*\_spill\_f: 5'-

AACTATGAGTTTAACTGCTG-3' and *pmoA\_spill\_r*: 5'-  
TCAAATTCGCACAATCTTT-3') were developed using Primer Express v 2.0.  
OPU1 enumeration was performed using cycling conditions as reported previously for  
the OPU3 *pmoA* phylotype. To enumerate the novel phylotype, a SYBR assay (Power  
SYBR green, Applied Biosystems) was developed. 1µl each of template DNA, forward  
and reverse primer (0.5µM final concentration), and water were added to a final volume  
of 20µl. qPCR was performed with a 95°C denaturation for 10 min, followed by 40  
cycles of 95°C (15'') and an extension at 58°C (1'). A final dissociation curve was  
included. For all functional gene assays, CsCl-purified linearized plasmid DNA was used  
as a standard. Primers displayed >95% amplification efficiency as measured over five  
orders magnitude and cross reactivity between target templates was below detection.  
Limit of detection for *pmoA* qPCR assays was 10 copies per reaction. The results were  
standardized to the mL of seawater.

#### *Statistics:*

A Wilcoxon Test was performed using Kaleidagraph (version 4.1) to determine the  
significance of the changes observed in qPCR results over the time.

#### *Clone libraries, sequencing, and phylogenetic analysis:*

*pmoA* gene fragments were amplified by polymerase chain reaction (PCR) from DNA  
obtained from eleven samples representing different time points and locations during our  
sampling campaign (Supplementary Table S2) using primer set wcpmoA189f and  
wcpmoA661r<sup>43</sup>. The PCR protocol consisted of an initial denaturation of 3 min at 94°C,

139 followed by 28-40 cycles of 1 min at 94 °C, 30s at 56 °C, and 45s at 72°C, ending with a  
140 final extension of 5 min at 72 °C. The PCR master mix consisted of of 1-20 ng/μl DNA  
141 and either 1X GoTaq Flexi Reaction Buffer, 1.25 u of GoTaq Flexi DNA Polymerase  
142 (Promega Corporation, Madison, WI), 2.5 mM MgCl<sub>2</sub>, 0.25 mg/ml BSA, 100 μM dNTP  
143 mix, 1μM of each primer, and ddH<sub>2</sub>O to a final volume of 25 μl; or OmniTaq DNA  
144 polymerase (0.5 μL), 1X Omni Taq reaction buffer, 100 μM dNTP mix, 1μM of each  
145 primer and ddH<sub>2</sub>O to a final volume of 25 μl.

146       Amplified *pmoA* gene fragments were gel-purified using the QIAGEN Qiaquick  
147 gel extraction kit (Qiagen, Santa Clarita, CA, USA), and cloned into a pCR4-TOPO  
148 plasmid vector (Invitrogen, Inc., Carlsbad, CA, USA) at a 1:3 vector to insert ratio. The  
149 ligation products were transformed into *E. coli* One Shot Top10 competent cells  
150 (Invitrogen, Inc., Carlsbad, CA, USA). Ampicillin resistant clones were selected and  
151 grown up overnight at 37 °C in freezing medium (LB + 100 μg/ml Amp + 10% glycerol).  
152 One mL of the clones was spun down and cell pellets were sent to Laragen, Inc (Culver  
153 City, CA) for Sanger sequencing. Sequences were also screened for closest relatives  
154 using the blastn application of the NCBI database. Sequences were aligned using Clustal  
155 X version 2.1 and manually adjusted using Seaview<sup>44</sup>. Operational taxonomic units  
156 (OTUs) were defined as sequences sharing more than 97% similarity and were calculated  
157 using MOTHUR<sup>45</sup>.

158 Sequences were translated using the online tool EMBOSS Transeq  
159 (<http://www.ebi.ac.uk/emboss/transeq/>). The amino acid sequences were aligned with  
160 Clustal X version 2.1 and manually adjusted using Seaview<sup>44</sup>. Phylogenetic distances  
161 were calculated using the Observed Divergence matrix, and the neighbor joining method

was used to evaluate tree topologies. Phylo\_win version 2.0 was utilized to plot tree topologies<sup>44</sup> whose robustness was tested by bootstrap analysis (1000 resamplings).

### ***Supplementary Discussion***

#### ***Evidence that Macondo Methane was Dispersed through the Water Column after June 2010:***

The methane present in the water column at MC118 in June 2010 was distinct from the thermogenic methane that typifies the MC118 site (Bowles et al. 2011, *Geochimica et Cosmochimica Acta*). The mid-water methane concentration measured at MC118 in June 2010 had a  $\delta^{13}\text{C-CH}_4$  of  $-53.8 \pm 1.5 \text{ ‰}$  ( $n=5$ ) and was as depleted as  $-55 \text{ ‰}$ , which is significantly lighter than the average  $\delta^{13}\text{C-CH}_4$  signature of methane at MC118 ( $-45 \text{ ‰}$ ) and outside the bounds of the range noted by the reviewer ( $-48$  to  $-52 \text{ ‰}$ ). We have made nearly 100 measurements of methane concentration and isotopic composition in the water column above MC118 since 2010. Typically, methane concentrations are maximal near the seafloor and decrease with distance above the bottom. The concentrations measured in June 2010 were low except for the anomalous high concentration zone present in the mid-water; so we conclude that this methane was Macondo in origin.

Furthermore, other stations north of MC252, and east/northeast from MC118 also exhibited methane concentration  $>100 \text{ nM}$  after July 2010. For example, we observed a methane concentration of  $109 \text{ nM}$  at  $750 \text{ m}$  at Cape Hatteras Station 68 (29.04, -88.12). This station is located  $23 \text{ nm}$  from MC252, which lead us to conclude that deepwater plume methane was dispersed upwards through the water column in a broad fashion.



185 Other examples of stations east/northeast of MC252 that exhibited methane  
186 concentrations >100 nM are presented in a table on the following page.  
187

				Distance to	Heading	189	
				MC252	from		
Site/				(nautical	MC252	Depth	Methane
Station	Date	Lat	Long	miles)	(degrees)	(m)	(nM)
CH065	11 Sept 2010	28.71	-87.93	24	94	309	138
CH066	12 Sept 2010	28.88	-87.93	26	70	719	132
CH066	12 Sept 2010	28.88	-87.93	26	70	567	157.
CH066	12 Sept 2010	28.88	-87.93	26	70	444	125
CH067	12 Sept 2010	29.04	-87.93	30	52	51	143
CH068	12 Sept 2010	29.04	-88.12	23	37	750	109
CH076	13 Sept 2010	29.24	-88.01	35	33	68	174
CH077	13 Sept 2010	29.23	-87.82	42	44	212	125
CH077	13 Sept 2010	29.23	-87.82	42	44	186	141

*Data Availability:*

All of the data presented in this paper are available electronically through the Gulf of Mexico Research Initiative's Data Warehouse (<https://data.gulfresearchinitiative.org/>).

**Supplementary References**

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## Captions for Supplementary Figures

**Supplementary Fig. 1. Map showing the study sites over space and time.** The sampling sites as a function of time since the Deepwater Horizon exploded are shown (colour code denotes time). The star denotes the location of the Macondo wellhead.

**Supplementary Fig. 2. Average methane concentration over 50-day periods.**

Maximum methane concentrations over time, binned in 200m depth and 50 day intervals (colour coding is the same as Fig. 1) illustrating dispersion of the deep-water plume over time.

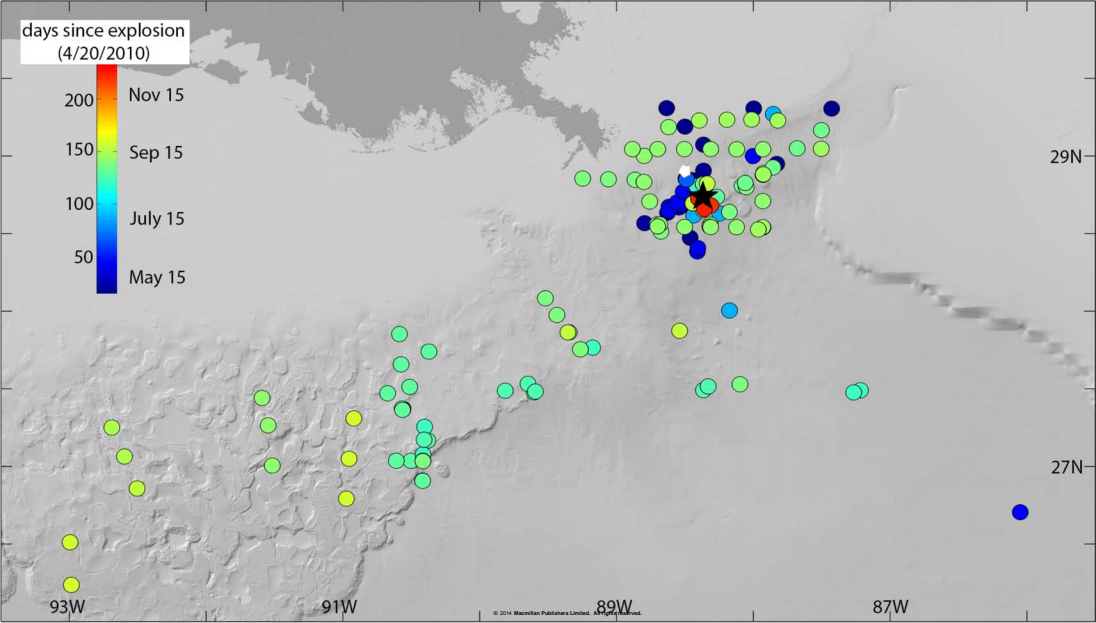
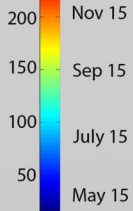
**Supplementary Fig. 3. Dissolved alkane concentrations in the water column during May and June 2010.** Concentration of C<sub>2</sub>-C<sub>5</sub> alkanes (time colouring extends to day 50 only as concentrations were below detection after that time; colour coding same as Figs. 1.

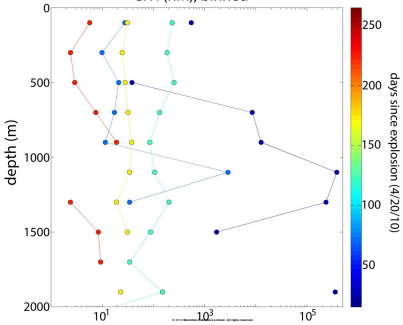
**Supplementary Fig. 4. Dissolved nutrient concentrations in the water column over the 10-month time-course.** Concentration of ammonium, nitrate+nitrite, and phosphate over depth (all concentrations are in µM) for the various study sites are shown.

Supplementary Table 1.

Date	Research Vessel	Site/ Station	Lat	Long	ID	Depth (m)	Extraction Method	Remarks
12-May-10	Pelican	Site_39	28.70	-88.39	WC45	1265	MoBio Kit	Combined DNA
14-May-10		Site_33A	28.72	-88.42	WC75	1220	MoBio Kit	
14-May-10	Pelican	Site_34A	28.71	-88.39	WC65	1020	MoBio Kit	Individual sample
26-May-10	Walton Smith	WS2	28.73	-88.41	C4-9	1120	MoBio Kit	Combined DNA
4-Jun-10		WS6	28.74	-88.38	C73-3	1180	MoBio Kit	
30-May-10	Walton Smith	WS36	28.71	-88.41	C40-6	1220	Vetriani et al., 1999	Individual sample
1-Jun-10	Walton Smith	WS53	28.73	-88.38	C59-3	1170	Adams et al., 2013	Individual sample
9-Sep-10	Oceanus	022.01	29.00	-88.80	179	162	MoBio Kit	Individual sample
30-Aug-10	Oceanus	014.02	27.37	-90.56	101	1222	MoBio Kit	Individual sample
30-Aug-10	Oceanus	014.02	27.37	-90.56	102	1000	MoBio Kit	Individual sample
30-Aug-10	Oceanus	014.02	27.37	-90.56	103	700	MoBio Kit	Individual sample
13-Sep-10	Oceanus	027.11	28.55	-88.32	242	1289	MoBio Kit	Individual sample

days since explosion  
(4/20/2010)

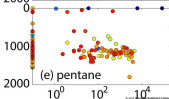
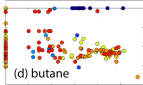
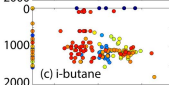
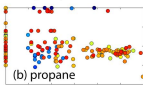
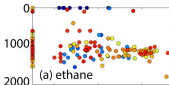


CH<sub>4</sub> (nM), binned



concentration (nM)

depth (m)



$10^0$   $10^2$   $10^4$

40  
30  
20  
days since  
4/20/10

